

A Chromosome RNAissance

Minireview

Abby F. Dernburg^{1,3} and Gary H. Karpen^{2,3}

¹Life Sciences Division
Lawrence Berkeley National Lab
One Cyclotron Road
Berkeley, California 94720

²MCBL
The Salk Institute
10010 North Torrey Pines Road
La Jolla, California 92037

In RNA-mediated interference (RNAi), double-stranded RNAs (dsRNAs) target complementary mRNAs for degradation. New work demonstrates that essential chromosomal functions are mediated through RNAi protein components and short RNAs, which alter chromosome function at specific DNA loci via histone modification.

New roles for RNA in regulating gene expression have recently been revealed by the discovery of RNA interference (RNAi), which has rapidly become a standard experimental tool for targeted destruction of mRNAs in worms, flies, plants, and mammals. The molecular mechanism of RNAi involves proteins of the Dicer family, which cleave dsRNAs to generate small interfering RNAs (siRNAs) that range from 21 to 28 nucleotides, depending on the organism (reviewed by Hannon, 2002). siRNAs target the RNA-induced silencing complex (RISC) to degrade homologous mRNAs, resulting in loss of specific protein expression. siRNA production can also be amplified by RNA-dependent RNA polymerases (RdRPs), which synthesize new dsRNAs using mRNA templates and siRNA primers. Proteins essential for the degradation pathway, including Dicer and Argonaute, are highly conserved in fungi, plants, and animals, although absent in budding yeast. These proteins have been shown to play essential roles in organismal development, germline fate, and host defenses against transposable elements and viruses.

The inheritance of genetic traits and the organization of genomic information in eukaryotes have been generally regarded as DNA-mediated functions. However, surprising new results demonstrate that RNA plays essential roles in epigenetic inheritance and chromosome function in *Schizosaccharomyces pombe* and *Tetrahymena thermophila*. In each of these processes, small RNAs appear to target regulatory proteins, including enzymes that modify histone tails, to specific, homologous chromosomal loci, using protein components of the RNAi machinery.

Establishment of Silencing in *S. pombe*

Genes inserted into centromeric regions or at the silent mating type locus (*mat 2/3*) are silenced in *S. pombe* by the local recruitment and spreading of proteins including Ctr4, Swi6, and Rik1. Swi6 is recruited to these loci by

binding of its chromodomain to a particular posttranslational mark on a histone tail, methylated lysine at position 9 of histone H3 (Me(Lys9)H3). This modification has been linked to heterochromatin establishment and function in many organisms. Swi6 can multimerize and also interacts directly with Ctr4, the histone methyltransferase (HMT) that adds methyl groups to H3 K9, providing a mechanism for spreading the methylated heterochromatin. A clear link between centromeric silencing and chromosome segregation was forged by the demonstration that Swi6 is necessary for the recruitment of cohesin, a protein that maintains contact between sister chromatids from S phase until anaphase (Bernard et al., 2001; Nonaka et al., 2002).

A role for RNAi machinery and siRNAs in fission yeast centromeric silencing has been demonstrated in two papers recently published in *Science*. Martienssen, Grewal, and coworkers demonstrated that mutations in Dicer (*Dcr1*), Argonaute (*Ago1*), and RdRP (*Rdp1*) eliminated silencing of a *ura4+* gene inserted into the outer and inner repeats that flank the central core of the centromeres, as previously observed for *swi6* and *ctr4* mutations (Volpe et al., 2002). A corresponding reduction in Me(Lys9)H3 was observed in the outer repeats of these mutant cells. These authors searched for candidate RNAs that might mediate silencing and made the remarkable discovery that large noncoding RNAs (~1.4 and 2.4 kb) homologous to these centromeric repeats accumulated in *dcr1*, *ago1*, and *rdp1* mutant cells but were not detected in wild-type cells. Nuclear run-on assays demonstrated that these RNAs were transcribed in wild-type cells but were highly unstable. This result suggests that the long RNAs are synthesized independently of the RNAi machinery and are rapidly diced into siRNAs. Direct evidence for this idea was provided by Reinhart and Bartel (2002), who cloned small RNAs of the structure predicted for Dicer products from *S. pombe* and recovered abundant species homologous to the centromeric repeats. Volpe et al. also provided evidence that Rdp1 protein is physically bound to the outer repeats, using a chromatin immunoprecipitation (ChIP) assay. Taken together, these results suggest that Rdp1 within the outer repeat chromatin may transcribe the second strand from nascent centromeric transcripts, generating dsRNAs. Dcr1 would process these dsRNAs into siRNAs, resulting in local H3-K9 methylation and silencing (Figure 1). Methylation could be targeted to the repeats by direct recruitment of the Ctr4 HMT or a histone deacetylase activity (HDAC) such as Ctr3, which would remove the acetyl modifications that inhibit methylation. Further experiments should illuminate the pathway leading from siRNAs to histone methylation, and also the contribution of Ago1 to this process.

A fundamental question generated by this work is whether the RNAi machinery is necessary to establish silencing in *S. pombe*, to maintain it once established, or both. In a related paper, Grewal and colleagues (Hall et al., 2002) demonstrate that normal silencing at the mating-type locus (*mat 2/3*) also relies on the RNAi proteins Dcr1, Rdp1, and Ago1, as well as *cis*-acting DNA

³Correspondence: afdernburg@lbl.gov (A.F.D.), karpen@salk.edu (G.H.K.)

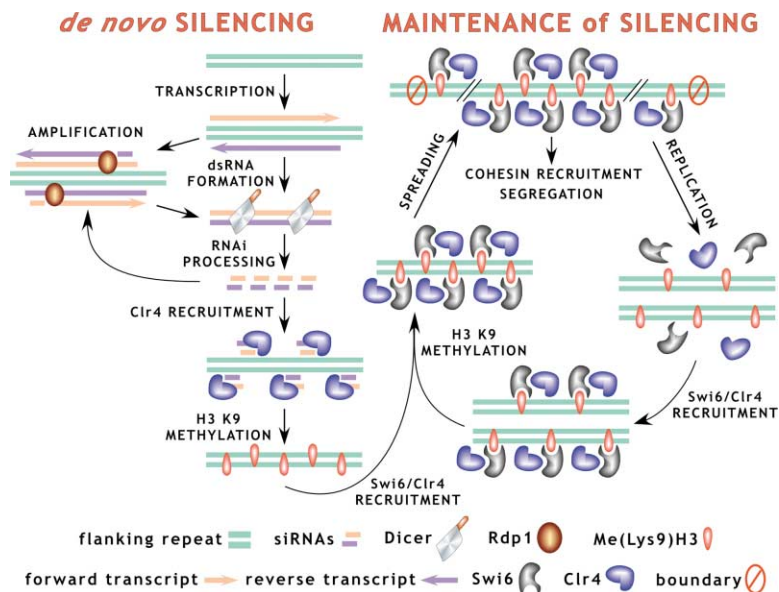


Figure 1. Models for the Establishment and Maintenance of Silencing in *S. pombe*

Silencing initiates at the *mat* loci and centromere flanks in response to dsRNA and siRNA production, which may be amplified by the tethering of Rdp1. The siRNAs lead to methylation of H3-K9 at sites of homologous DNA, possibly by direct recruitment of Ctr4 (as shown), or perhaps more indirectly by recruitment of other activities. The RNAi machinery is dispensable for maintenance of silencing. Epigenetic inheritance and spreading of the silent state is most likely mediated by segregation of modified nucleosomes to daughter strands during replication, recruitment of Ctr4 (and Rik1) by Swi6 binding to Me(Lys9)H3, and modification of new, adjacent nucleosomes in the region. Spreading proceeds until an “insulator” or “boundary element” is encountered.

sequences homologous to the centromere repeats. Nevertheless, a previously silenced *mat* locus retains high levels of Me(Lys9)H3 and silencing through many rounds of replication and division in RNAi-deficient cells. Thus, maintenance of the silenced state at the *mat* locus does not require RNAi machinery and is most likely inherited through propagation of Me(Lys9)H3 and/or Swi6 during replication (Figure 1; Nakayama et al., 2000). Hall et al. also demonstrate that RNAi-deficient cells cannot effectively establish silencing at the *mat* locus even with normal levels of Ctr4 and Swi6. In the absence of Swi6, Me(Lys9)H3 is restricted to the immediate vicinity of the putative RNA template, suggesting that it acts as a silencing “initiation” site. Normally, silencing then spreads over a 20 kb region and is halted by “insulators” or “boundary elements.” In summary, this work shows that Swi6 is necessary for reinforcement and spreading of the Me(Lys9)H3 mark but is dispensable for its initial establishment. Conversely, RNAi is required at the *mat* locus to establish silencing, but not to maintain it.

A difference between centromeric and *mat* silencing is that at the *mat* locus there are no obvious defects in RNAi-deficient cells unless silencing is experimentally erased, either by genetic introduction of a Me(Lys9)H3-free copy of the locus or by treatment of cells with trichostatin A (TSA), a histone deacetylase inhibitor. By contrast, centromeres become desilenced in RNAi-deficient cells, raising the interesting possibility that reestablishment of silencing is more critical on an ongoing basis at the centromere than at *mat*.

DNA Elimination in *Tetrahymena*

Unlike most other single-celled organisms, *Tetrahymena* and other ciliates have two types of nuclei. Germ-line micronuclei (MICs) contain a complete copy of the genome but do not express genes. Somatic macronuclei (MACs) are transcriptionally active, but their development from micronuclei involves massive chromosome rearrangements through DNA elimination, the excision of abundant dispersed sequences called internal eliminated sequences (IES) and breakage eliminated se-

quences (BES) (reviewed by Yao et al., 2002). During the sexual cycle (conjugation) of *Tetrahymena*, two cells, each containing a MIC and a MAC, fuse. The MICs in each cell undergo meiosis, and the cells exchange haploid meiotic products, which fuse to form a zygotic nucleus. The zygotic nucleus divides twice to produce four nuclei; two are retained as MICs, and two undergo DNA elimination leading to the development of new MACs. Several genes required for normal MAC development have previously been identified, including three PDD (programmed DNA degradation) genes.

The Gorovsky group (Mochizuki et al., 2002) found that Twi1p, a Piwi family protein related to Argonaute, is also essential for successful conjugation, IES elimination, and MAC development. They hunted for RNAs of a size similar to siRNAs and detected them at the stages of conjugation preceding DNA elimination. These “scnRNAs” (or “scan RNAs,” see below and Figure 2) are not detected in TWI1 knockout cells and are delayed in their appearance in PDD1 knockouts. The sequences of the small RNAs have not been directly determined, but they hybridize far more strongly with MIC DNA than with the MAC genome, indicating that they correspond to IES and/or BES sequences. Some of these RNAs may be derived from IES-containing transcripts detected during early MIC meiosis (Chalker and Yao, 2001). During conjugation, Twi1p is first observed in the cytoplasm, then in the parental MAC, and finally in the new MAC. Together with the observation of small RNAs and results from previous studies, this localization pattern suggested that Twi1p could be transmitting RNA-encoded information from the old to the new MAC to designate the sequences to be eliminated.

In a complementary study by Allis and coworkers (Taverna et al., 2002), elimination of the IESs was shown to be associated with Me(Lys9)H3, the identical modification targeted to *S. pombe* centromeres and the *mat* locus by the RNAi machinery. Pdd1p and Pdd3p, two gene products essential for elimination, contain chromodomains, which were shown to bind in vitro to Me(Lys9)H3.

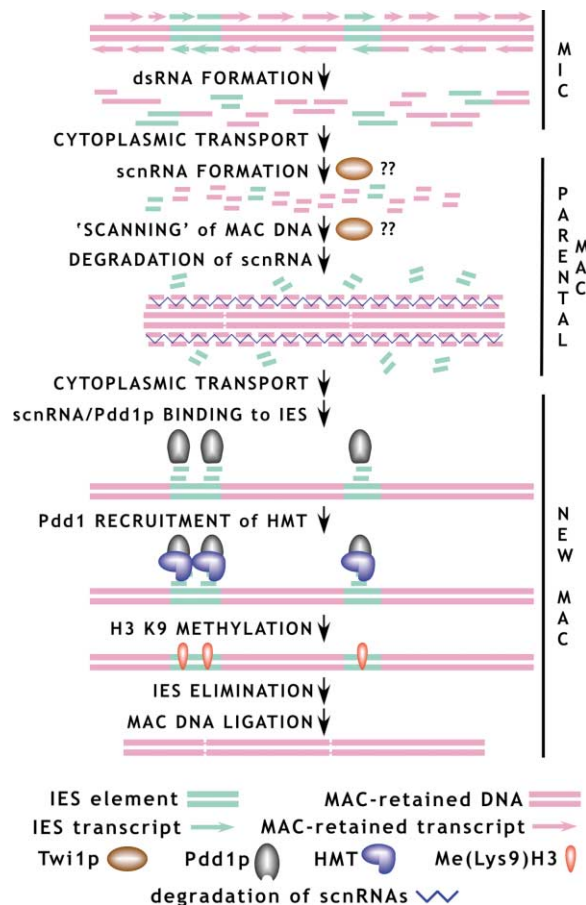


Figure 2. A Model for DNA Elimination in *Tetrahymena*

This is an adaptation of the model of Mochizuki et al. (2002), in which dsRNAs homologous to the entire genome are produced in the micronuclei (MIC) and transferred through the cytoplasm to the parental MACs. A suggested function for Twilp is to mediate a "scanning" process in the parental MAC, in which RNAs corresponding to MAC sequences are recognized and degraded. Intact scnRNAs homologous to the MIC-specific IESs (and BESs) are then transported to the new MAC, where scnRNAs or the resulting Me(Lys9)H3 mark recruits Pdd1p and other components required for elimination to the IES/BES sequences.

Western blotting and immunolocalization demonstrated that Me(Lys9)H3 is only present at the time and place where elimination occurs. ChIP analysis revealed that Pdd1p and Me(Lys9)H3 are associated preferentially with eliminated sequences in the developing new MAC. Furthermore, abnormal PDD1 expression strongly reduces but does not eliminate Me(Lys9)H3. Finally, the sufficiency of Pdd1p to promote elimination was demonstrated in an elegant experiment in which the protein was artificially tethered to an ectopic genomic locus. We do not yet know whether Pdd1p mediates K9 methylation, or if Pdd1p localization is normally downstream of methylation.

Mochizuki et al. propose a model (Figure 2) in which RNAs originating in the MIC are first transported to the parental MAC. These RNAs somehow "scan" the genome for homologous DNA. RNAs matching DNA sequences are degraded, so that only RNAs *not* homo-

gous to the genome of the old MAC are retained and transported to the new MAC. Taking the two papers' results together, we can incorporate the idea that the scnRNAs mediate K9 methylation and Pdd1p recruitment, ultimately leading to elimination. Me(Lys9)H3 is not detected in vegetative cells, indicating that this epigenetic mark is implemented *de novo* during conjugation. The requirement for the RNAi machinery to establish but not to maintain silencing at the *mat* locus in *S. pombe* is thus consistent with its proposed role in IES elimination in *Tetrahymena*.

siRNA Recruitment of Silencing and Elimination Proteins

Fundamental questions remain about the role of siRNAs in targeting histone modification enzymes to particular loci. Substrates for modification are likely specified by base-pairing interactions. Small RNAs could be targeted to chromosomes by base-pairing with nascent transcripts, much as they are targeted to processed mRNAs. If so, the targeting machinery might share components with RISC. Alternatively, small RNAs could associate directly with DNA. These associations could be mediated by proteins containing chromodomains, some of which bind both RNA and histone tails *in vitro* (Akhtar et al., 2000; Muchardt et al., 2002) and require an RNA component for heterochromatin localization *in vivo* (Maison et al., 2002). However, some chromodomain proteins, including Swi6, act downstream of RNA targeting in silencing pathways. Base-pairing interactions between small RNAs and complementary RNA and/or DNA might also be mediated by the enigmatic Argonaute/Piwi family.

Events downstream of H3-K9 methylation could be specified by recruitment of other activities directly by RNA, by differences in the Me(Lys9)H3 binding proteins present in different nuclei, by sequestration of particular loci into specialized nuclear compartments, or by combination of Me(Lys9)H3 with other modifications that specify the "histone code" (Jenuwein and Allis, 2001). Future studies should elucidate how an identical chromatin mark—Me(Lys9)H3—can mediate very distinct outcomes, including silencing and DNA elimination.

Transcription of siRNA Precursors

A paradox arises from observations that the silencing of specific DNA sequences is caused by production of transcripts from within these very regions. It seems that chromatin-mediated repression would eventually prevent production of siRNA precursors and thereby silence the silencing mechanism. However, Volpe et al. demonstrate that transcription continues from the centromere repeats of silenced cells. Other RNAi-mediated phenomena, particularly cosuppression, in which genes are silenced by the presence of extra copies of homologous DNA sequences (reviewed by Zamore, 2002), also appear to require ongoing transcription from "silent" loci. The continued production of regulatory transcripts from silenced regions could simply result from a feedback mechanism, consistent with indications that Swi6 may repress forward strand transcription of the *S. pombe* outer repeats (Volpe et al., 2002). Perhaps only occasional nascent transcripts are necessary for RNAi-mediated silencing. Evidence for physical association of an RdRP with the *S. pombe* centromeric repeats suggests that rare transcripts could be immediately utilized to

make dsRNA, thus amplifying siRNA production (Figure 1). A critical unanswered question is how RdRPs are recruited to specific DNA sequences and whether recruitment requires Me(Lys9)H3. Finally, regulatory transcripts destined for processing into siRNAs might also be synthesized by RNA polymerases, such as Pol I or III, which may be relatively insensitive to chromatin-mediated silencing mechanisms. Identification of the promoters and polymerases that regulate transcription of centromeric repeats in *S. pombe* and at other silent loci should address these issues.

Repetitive Sequences and RNAi

Diverse silencing mechanisms are induced by repetitive DNA, including cosuppression in nematodes, repeat-induced point mutation (RIP) in *Neurospora*, and position-effect variegation (PEV) in *Drosophila*. In cases where RNAi components have been implicated in repeat-induced silencing, it has been hypothesized that the trigger might be aberrant coding mRNAs (reviewed by Zamore, 2002). Alternatively, transcripts might be shunted into the RNAi pathway through the recruitment of RdRPs to repetitive DNA, if indeed this is a general phenomenon.

If repetition is a trigger for silencing, how do essential repeated genes escape silencing? The ribosomal RNA genes are very actively transcribed by Pol I, but it is notable that Pol II genes inserted into these repeats are silent (Huang, 2002). Repeated genes could bypass silencing by sequestration in specialized compartments (e.g., the nucleolus) or by utilizing promoters for polymerases that may be insensitive to silencing. Essential noncoding transcripts may also be designed to avoid processing by the RNAi machinery, perhaps by differences in 5' capping or polyadenylation.

How did repeat-associated silencing mechanisms evolve? The centromeric repeats of *S. pombe* and the IESs of *Tetrahymena* are thought to have originated from transposable elements; for example, the *S. pombe* centromeric repeats bind to cellular proteins with homology to transposases (Nakagawa et al., 2002). It is likely that the maintenance of tandem and dispersed repeats as heterochromatin is an adaptation of defense mechanisms that protect the host from transposon invasion or mobilization. In some cases, these silencing mechanisms appear to be specific to the germline, where transposon mobilization and ectopic recombination between repeats are especially hazardous. The recent work reviewed here has illustrated how eukaryotes may have coopted defensive mechanisms to mediate essential functions at specialized repeats, including silencing of genetic information, sister-chromatid cohesion, and programmed DNA rearrangements.

What properties of transposons and other repeats trigger silencing? It is possible that the silencing mechanisms recognize unusual structures present in repeated DNA as "dangerous," rather than the abundance of sequences per se. Although transposons and retrotransposons are usually present as dispersed repeats, the inverted or direct repeats at their termini may flag them for silencing. Alternatively, promoters within the transposons may trigger silencing. A role for abundance is suggested by the observation that multiple, dispersed copies of "normal" genes induce RNAi-mediated silencing in *Drosophila* (Pal-Bhadra et al., 2002). However,

silencing in this case could be instigated by the P element ends flanking the integrated constructs. Additional studies are necessary to distinguish between the effects of repeat structure and sequence abundance in RNAi-mediated silencing.

RNAi and Chromosome Function

These recent publications link RNAi machinery and siRNAs to epigenetic chromatin modifications and chromosome behavior. They represent major advances in our understanding of how particular sites are marked for heterochromatin formation and will likely enhance the status of "junk" DNA by demonstrating unexpected roles for specific noncoding sequences and repeated DNA. It is easy to imagine that RNAi-related mechanisms might contribute to a wide diversity of chromosome behaviors, including X chromosome inactivation in mammals, the "spreading" of silencing through the pericentromeric repetitive DNA of higher eukaryotes, telomere maintenance, sex chromosome silencing in the germline of many species, hybrid dysgenesis in *Drosophila*, chromatin diminution in *Ascarid* nematodes, and nucleolar dominance in plants. These exciting new developments should stimulate a "RNAissance" of investigations into these and many other enigmatic chromosome phenomena.

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